ASSESSMENT OF CIGUATERIC FISH IN HAWAII BY IMMUNOLOGICAL, MOUSE TOXICITY AND GUINEA PIG ATRIAL ASSAYS

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Ciguatera studies were determined at the Waianae Boat Harbor, Oahu when 12 or more individuals became ill after eating freshly caught mullet (*Mugil cephalus*, amaama) in January–March,1991. Typical clinical manifestations of ciguatera were shown by the patients. Immunological assay for ciguatoxin and polyethers with monoclonal anti-ciguatoxin (MAb-CTX) showed 80% of the mullet to be borderline to positive. The herbivores, *Ctenochaetus strigosus* (kole), *Acanthurus sandvicensis* (manini) and other *Acanthurus* sp. (palani), showed high levels of toxins. The mackerels showed little or no toxic levels, while the carnivores (jack, amberjack) showed borderline to positive toxicity. Abundant green algae (*Bryopsis*), 30–60cm below the seawater surface, found at all five sites examined, contained *Gambierdiscus toxicus* in moderate numbers. At 2 sites, when *Bryopsis* disappeared (summer - early winter), no *Gambierdiscus toxicus* was found. Fish extracts of mullet and other herbivores (palani, manini, kole-surgeonfishes) were highly toxic to mice. Guinea pig atrium analysis of the wild *Gambierdiscus toxicus* and fish extracts showed typical ciguatoxin-like inotropic response strongly inhibited by tetrodotoxin.

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In January–March,1991, an outbreak of ciguatera poisoning occurred among individuals eating mullet (*Mugil cephalus,* amaama) from Waianae Boat Harbor. Approximately 12 people exhibited classical symptoms of ciguatera poisoning (Hokama,1988) with an older couple hospitalized for several days. Based on knowledge of the ciguatera food chain (Randall, 1958; Hokama et al.,1986; Hokama et al.,1993), a systematic study of Waianae Boat Harbor was carried out and included:

1.Examination of algae species;

2.Examination of ciguatoxin producing *Gambierdiscus toxicus*; 3.Immunological testing of as many species of fish from the boat harbor;

4.Analysis of alga and *G. toxicus* chemical extracts for ciguatoxin by mouse toxicity bioassay and by the guinea pig atrium assay;

5.Analysis of fish extracts using the mouse toxicity and guinea pig atrium assays; and

6.Water quality data (from the Department of Health).

Basis for this systematic assessment is that of Yasumoto et al., (1979,1980,1984) and Hokama et al. (1993).

METHODS

SURVEY AREA

The Waianae Boat Harbor (117057m²) includes 4 ramps and 3 docking areas for boats. Two water inflow outlets are the entrance channel to the ocean, and a tunnel located between the boat docks (fresh water and seawater runoff). Five areas are surveyed within the boat harbor (Fig.1).

ANALYSIS FOR *G. TOXICUS*

Algae specimens (0.5kg) were collected in a 5 litre ziplock bag containing 1 litre of seawater. The contents were shaken for 2 minutes to loosen epiphytic dinoflagellates from the alga. The salt water–algal suspension was passed through a 125μm sieve to remove larger algal fragments and then through a 37μm sieve. This residue was backwashed with a filtered seawater media, transferred into a 100ml sterile glass bottle and capped loosely to provide aeration. After gently shaking the algal sample bottle, 1.0ml was removed and transferred onto a Sedgewick Rafter Cell Counting Slide. Cell counts were carried out in tripli-
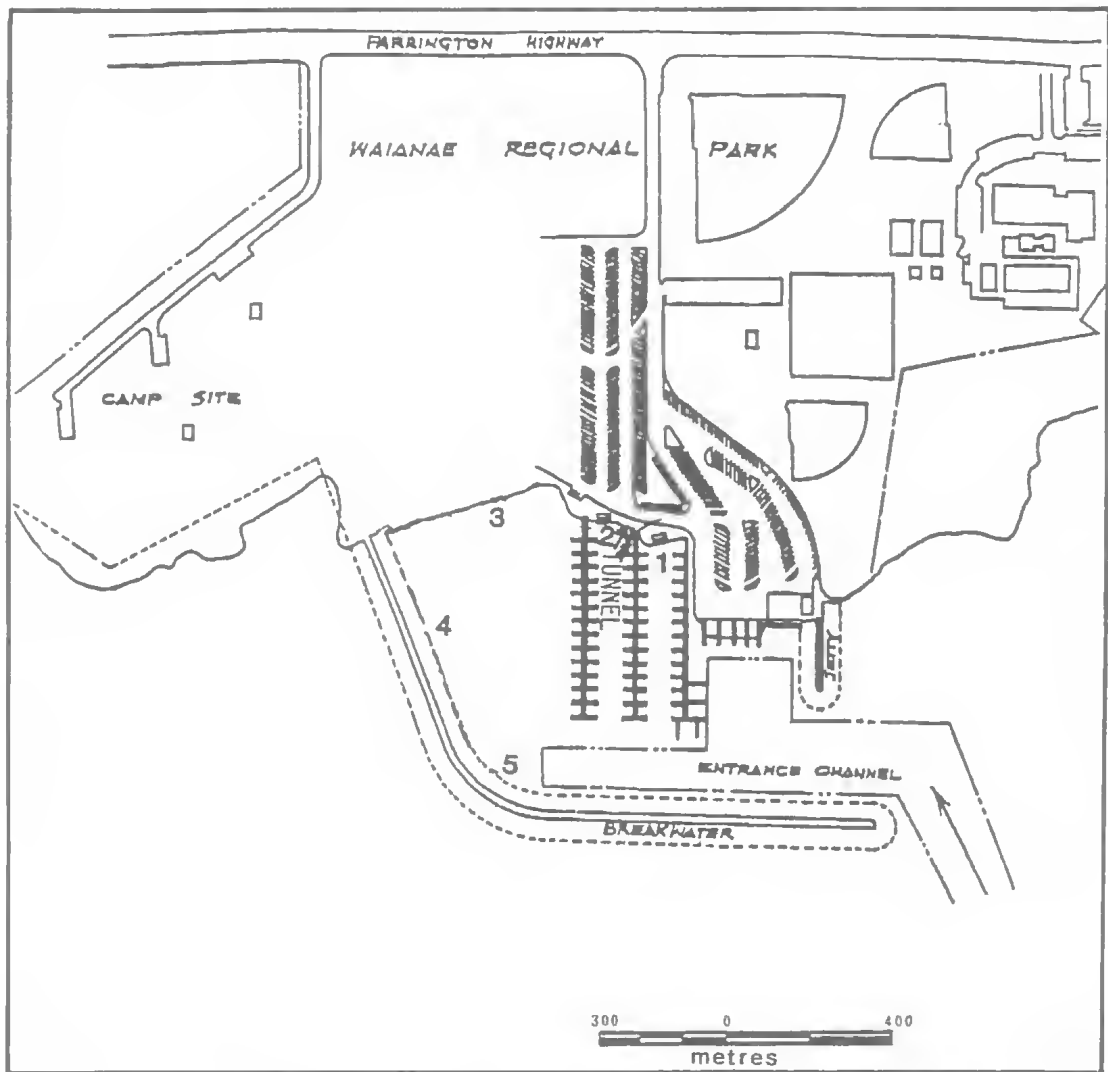
FIG. 1. Map of the survey area of Waianae Boat harbor (WBH). Tunnel is in the area of Section 2.

cate and the average number of cells determined per millilitre.

COLLECTION OF FISH

Fish samples (Table 4) were taken by divers mainly using spear-guns but also nets and lines and were identified from Tinker (1982).

FISH GUT SMEARS

An incision was made in the belly of the fish, in particular mullet (amaama), surgeonfish (kole) and Sandwich Island surgeon fish (manini) to extract gut contents which were mixed with 0.1 ml

of 0.85% NaCl and one drop was smeared onto a slide. The mixture was air dried and analyzed for *G. toxicus* with a microscope (Zeitz) at x400. Presence of other algal fragments were noted.

STICK ENZYME IMMUNOASSAY (S-EIA)

The S-EIA procedure comprised: a, make incision in fish tissue; b, insert skewered liquid paper-coated end of bamboo stick into flesh; c, air dry coated end of stick; d, immerse coated end of stick into absolute methyl alcohol for 0.5 sec; e, air dry; f, immerse in 1 ml monoclonal anti-ciguatoxin-horse radish peroxidase (MAb-CTX-

TABLE 1. Mouse toxicity assay scoring

Tox-icity	Description of Visible Clinical Symptoms in Mouse After Extract Injection
0	No ill effect
1	15-60 min: muscle contraction in lower back area (flexion), increased respiration, immobile (inactive), recovery.
2	Same as 1, but recover in 2-3 hours, pilo-erection
3	Recover in 12-24 hours: same as 2, muscle contraction, paralysis in extremities (usually hind legs), rapid and irregular breathing, immobile, closed eyes, pilo-erection, slight cyanosis (tail).
4	Symptoms as in 3, but death within 24*-48 hrs.
5	Symptoms of 3 and 4, death in less than 6 hrs.

*1 mouse unit, death within 24hrs of 20gm mouse; contains 7-9ng ciguatoxin in the sample of 100mg of crude extract/mouse injected (IP) (Hokama, personal estimation from Department of Health confirmed ciguateric fish extracts).

HRP); g, wash end of coated stick twice in buffered saline; h, immerse stick in horse-radish peroxidase (HRP) substrate; i, score color intensity of substrate as previously reported (Hokama,1988; Hokama et al.,1989). Final color: 0−1.2 is scored as negative, 1.3−1.9 borderline and 2.0 as positive. All fish scoring 1.3 are reported as rejections (not edible). S-EIA values

of 1.3 generally represent 0.4ng ciguatoxin or related polyether per gram of fish tissue.

MOUSE BIOASSAY (Kimura et al.,1982)

Swiss Webster mice weighing 20−25g were utilized in this study to assess the toxicity of fish extracts. 100mg of crude fish extract was resuspended in 1ml of 1% Tween 60 in saline and injected intraperitoneally (IP) into mice. Symptoms displayed by the mouse were observed at 30min, 1, 2, 4, 6, 8, 24 and 48hours after injection and rated on a scale of 0−5 according to toxicity (Table 1). One mouse unit equals 7−9ng of ciguatoxin per 100mg of crude extract which kills a mouse within 24hours. This is based on our estimation of ciguatoxin from known cases of ciguateric fishes obtained from the Department of Health.

GUINEA PIG ATRIAL ASSAY (Miyahara et al., 1989)

100mg of each fish extract was resuspended in 1ml of Krebs-carbonate solution. 100µl of the suspension was tested on the guinea pig atria. Subsequent inotropic and chronotropic actions were noted in addition to its pharmacological response to TTX (tetrodotoxin), verapamil and the adrenergic blockers (propranolol and phentolamine). The inhibitors, TTX, verapamil and adrenergic blockers, were given after the in-

TABLE 2.WBH Water Quality Physical Analysis and *G. toxicus* in 1992 (Bloom Year's Analysis).

		Sections*				
		1	2	3	4	5
Temp (°C)	mean ± S.D.	26.3 ± 0.85	26.2 ± 0.8	26.3 ± 0.8	26.2 ± 0.7	26.1 ± 0.7
	range	25.1-27.2	25.1-27.2	24.9-27.2	25.1-27.0	25.1-26.9
pH	mean ± S.D.	8.1 ± 0.2	8.1 ± 0.2	8.1± 0.3	8.1 ± 0.2	8.1 ± 0.2
	range	8.0-8.6	8.0-8.6	7.9-8.6	7.9-8.6	7.9-8.4
DO (mg/l)	mean ± S.D.	6.2±1.3	6.1±1.7	6.3±1.6	5.9±1.3	5.9æ0.8
	range	5.2-8.6	4.8-7.5	4.1-9.4	4.3-8.6	5.0-7.1
Salinity (%)	mean ± S.D.	3.5±0.4	3.5æ0.3	3.5±0.4	3.5±0.3	3.5æ0.3
	range	3.5-3.6	3.4-3.5	3.4-3.5	3.5	3.4-3.5
Conductance	mean ± S.D.	53.5±0.5	53.5±0.4	53.4±0.5	53.6æ0.3	53.6±0.4
	range	52.7-54.1	52.5-54.0	52.4-54.0	53.1-54.0	52.9-54.2
G. toxicus (cells/gm weight of alga)	mean ± S.D.	23.4±26.4	10±10.9	18.3±37.0	15.5±27.7	45.4±87.7
	range	0.9-65.6	0-26.5	0-120.3	1.3-90.7	0-268.9

*1.No relationship of physical properties of seawater to G. toxicus growth.
2.All values are means± S.D. for 9 months evaluation of each section (1 to 5).
3.The salinity in % of WBH generally below 4.0% maximum of seawater is due to the underground fresh water along the Leeward coast of Oahu.

otropic responses at 12.5μl of a 10^{-3}M concentration.

WATER QUALITY

Throughout Sections 1-5 (Fig.1), the salinity, temperature, pH, dissolved oxygen and conductance were measured utilizing a Surveyor 2 (Hydrolab Corp., Austin, Texas) (Table 2).

RESULTS

G. TOXICUS — DATA FOR 1992

Bryopsis growth was most prominent in Sections 3, 4 and 5 throughout the years. *Bryopsis* was generally sparse at Sections 1 and 2, between which the rainfall and wash water outlet is present. If non seawater enters, it is expected to lower the salinity and may interfere with algal growth. However, the lowering of salinity has not been observed in Sections 1 or 2 (Table 2). *G. toxicus* cell counts were best in all Sections on June 25,1992, especially Sections 3 and 5. October 29,1992 also showed moderate to high counts of *G. toxicus* in Sections 4 and 5.

It is suggested that constant presence of *Bryopsis* and *G. toxicus* helps to maintain the high toxicity level of herbivore fishes in the WBH (Table 4).

GUT SMEAR ANALYSIS

Gut smear analysis by microscope at x400 magnification revealed on several occasions, *G. toxicus* in smears of *Mugil cephalus* (mullet). This involved 3 samples of fish gut.

TABLE 3.Number of *Gambierdiscus toxicus*/gm of Alga in Waianae Boat Harbor at Various Time Periods and Stations

Dates (1992)	Station Number*				
	1	2	3	4	5
Mar. 27	1.4	2.1	0.0	3.2	4.0
Apr. 28	6.6	27.0	4.0	4.0	27.0
May 29	0.0	0.0	0.0	1.4	0.8
Jun. 25	15.0	20.0**	120.0	10.0	269.0
Jul. 24	NA	NA	3.2	2.5	8.0
Aug. 28	0.0	NA	2.7	0.0	16.0
Sep. 18	56.0	NA	17.0	30.0	4.0
Oct. 29	NA	NA	17.0	91.0	129.0
Nov. 25	NA	8.5	3.0	2.2	1.1

*NA, no *Bryopsis* available
**20 cells/gm *Ulva*, not *Bryopsis*

TABLE 4.Stick Enzyme Immunoassay (S-EIA) Evaluation of the Fish in Waianae Boat Harbor for Ciguatera (1992)

	No. of Fish	>2 Positive	1.3–1.9 Border line	<1.2 Negative
Ctenochaetus hawaiiensis (Hawaiian kole)	18	4 (22.2)	12 (66.7)	2 (11.0)
Acanthurus sandvicensis (Manini)	110	53 (48.1)	33 (30.0)	24 (21.8)
Mugil cephalus (amaama, mullet)	100	27 (27.0)	42 (42.0)	31 (31.0)
Kuhlia sandvicensis (aholehole)	51	18 (35.3)	18 (35.3)	15 (29.4)
Savotherodon sp. (Tilapia)	10	1 (10.0)	8 (80.0)	1 (10.0)
Acanthurus dussumieri (palani)	8	0 (0.0)	5 (62.5)	3 (37.5)
Abudefduf abdominalis (mamo)	7	0 (0)	7 (100)	0 (0)
Trachiurops crumenophthalmus (akule, halalu)	6	0 (0)	1 (16.7)	5 (83.3)
Caranx sp. (papio)	5	5 (100)	0 (0)	0 (0)
Mulloidichthys auriflamma (weke)	4	1 (25)	1 (50)	2 (25)
TOTALS	319	109* (34.2)	127* (39.8)	83 (26.0)

*positive plus borderline represent 75% of fish rejected (non-edible)

STICK ENZYME IMMUNOASSAY OF FISH

There is a high rejection rate of *Mugil cephalus* (Table 4) which is the species that caused the outbreak of ciguatera poisoning in the early months of 1992. Halalu (*Trachiurops crumenophthalmus*) was mostly negative (Table 4), and has never been implicated in ciguatera poisoning. This species has been continuously caught and eaten throughout 1991 and 1992 with WBH with no known incidence of ciguatera poisoning. Aholehole has shown a large number in the rejection category (Table 4), but no known toxicity has been reported. All the other species are not evaluated individually because of the numbers. Evaluation of all fish (67 in 1991) suggests a high prevalence of fish in the rejection category (borderline plus positive = 77.6%). This may be due to the continuous presence of *G. toxicus* in the

Mullet Flesh (pooled)

1-R

100 µl	TTX	Verap	Adren	Rinse
(100 mg/ml)	12.5 µl	12.5 µl	12.5 µl	
	(10^{-5} M)	(10^{-5} M)	(10^{-5} M)	
A	B	C	D	E

Mullet Gut

2-L

A   B   C   D   E

Mullet Flesh

3-R

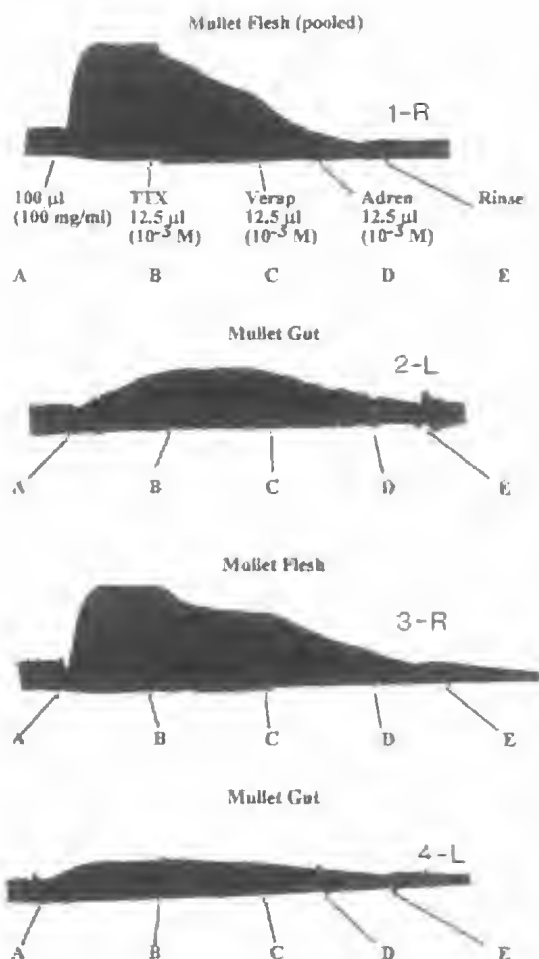A   B   C   D   E

Mullet Gut

4-L

A   B   C   D   E

FIG. 2. Guinea pig atrium assay with two mullet flesh and gut samples from WBH, 1992. 1-R; A, S-EIA positive pooled mullet flesh extract; B, addition of TTX; C, addition of verapamil; D, addition of adrenergic inhibitors; and E, rinse of physiobath with medium. 2-L; A, S-EIA positive pooled mullet gut extract; B, TTX; C, verapamil; D, adrenergic inhibitors; and E, rinse. 3-R; A, S-EIA positive pooled mullet flesh extract; B, TTX; C, verapamil; D, adrenergic inhibitors; and E, rinse. 4-L; A, S-EIA positive pooled mullet gut extract; B, TTX; C, verapamil; D, adrenergic inhibitors; and E, rinse. R=right atrium; L=left atrium.

enclosed WBH, which has only one outlet to the ocean. The non-toxicity of halalu suggest the significance of the food consumption habit and source of this species is different from the herbivores feeding on algae (*Bryopsis*).

S-EIA data for 1992 (Table 4) show rejection rates in descending order: mamo (100%), *Caranx*

sp. (100%), tilapia (90%), Hawaiian kole (87.9%), manini (78.2%), aholehole (70.6%), mullet (69%) and palani (62%). The samples of mamo, weke and *Caranx* sp. are too small to evaluate, though the high value of toxicity in *Caranx* sp. may be significant.

Mullet flesh and gut were all of high toxicity in all samples collected, except mullet flesh from 4/92. Surprisingly, extracts from negative mullets from 6/92 and 9/92 were highly toxic to mice. Similarly, Hawaiian koles were all highly toxic with mouse assay values of 5+. Manini flesh and gut extract showed variable results, although the majority of samples were highly toxic in mouse (5+). The 5/92 samples of *Caranx* gave a high toxicity value in the gut (5+) and a low toxicity value (2+) in the flesh. This is generally the pattern when examining flesh and gut separately in carnivores. Palani appeared to be of high toxicity in both flesh and gut extracts for samples on 3/92 and 6/92. Mamo and aholehole extracts gave comparable results with the flesh extract showing low toxicity (2+) and the gut extracts high toxicity (5+). Of interest is the high toxicity level of whole tilapia extracts obtained from mostly borderline tested fish in the S-EIA. Whether this is truly ciguatoxin (congeners) or other polyethers remains to be determined for the tilapia. The highly toxic gut extracts were essentially non-ciguatoxin-like in the mouse assay.

GUINEA PIG ATRIAL ASSAY

The results of the mullet tissue analysis (Table 5, Figs 2,3) suggest ciguatoxins as noted by the consistent inhibition of the inotropic effect by tetrodotoxin (TTX). See also the inotropic patterns presented for the mullet tissue extracts (Fig.2). In some cases, verapamil (verap.) also shows the inhibition of the Ca^{++} ion suggesting a maitotoxin-like toxin. The Hawaiian kole extracts showed variable inotropic response unlike the mullet, but with slight inhibition by TTX and verap. The manini flesh and gut extracts gave moderate inotropic responses with inhibition by both TTX and verap. This suggests possible ciguatoxin and maitotoxin-like toxins in these extracts. Though not shown here, a new toxin with sodium channel inhibition was observed in the guinea pig atrium assay. This toxin(s) was unlike TTX or PSP in solubility.

DISCUSSION

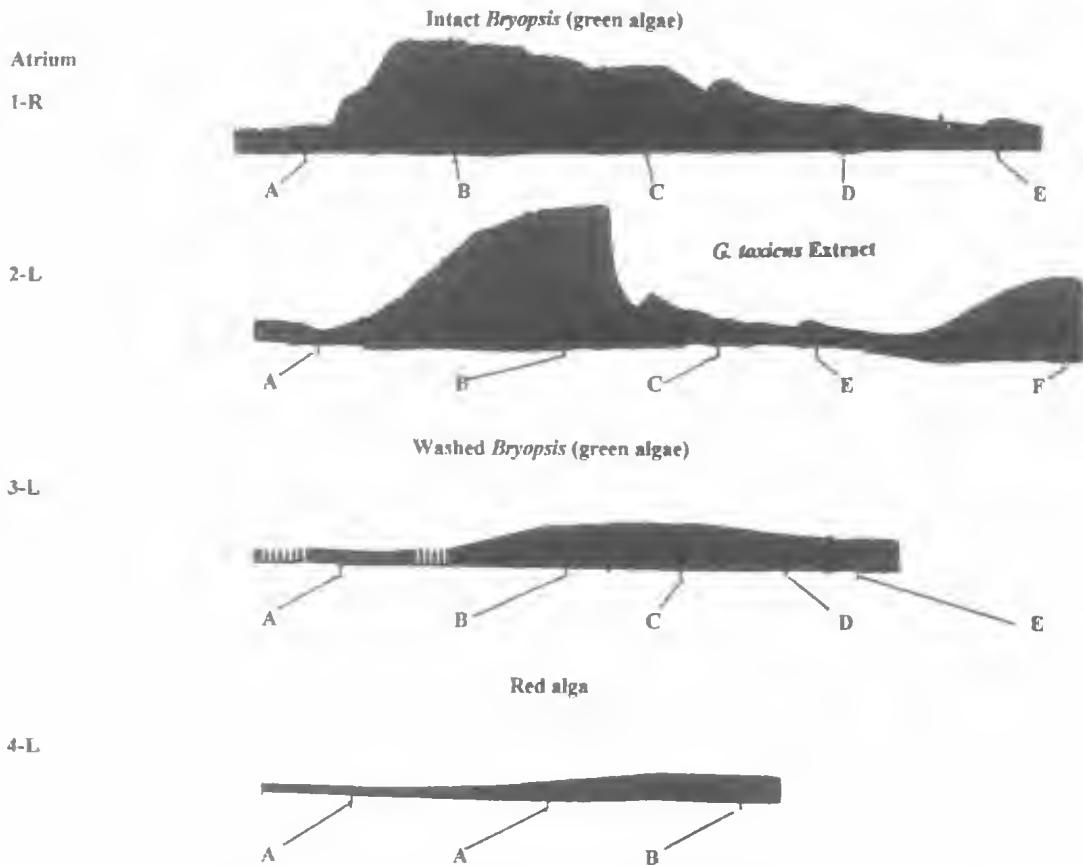The Waianae Boat Harbor appears to be an excellent model for the solution of possible en-

FIG. 3. Ciguatoxin in *Gambierdiscus toxicus* ciguatoxin-like toxins or no ciguatoxin in green and red algae. 1-R; A, 100μl of a 100mg/ml solution of intact *Bryopsis* acetone extract; B, addition of TTX (12.0μl of a 10^{-3} M solution); C, addition of verapamil (12.0μl of a 10^{-3} M solution); D, addition of a 12.0μl of a 10^{-3} M solution each of phentolamine and propranolol; and E, rinse with medium. 2-L; A, 100μl of a 100mg/ml solution of *G. toxicus* extract of a 22mg/ml solution; B, TTX added; C, verapamil added; E, rinse; F, return of inotropic response initiated by A. This indicates the CTX in *G. toxicus* extract remains tightly bound to atrium and the inhibitor (TTX) is removed by rinse. 3-L; A, 100μl of washed *Bryopsis* added (concentration 79 mg/ml, B, TTX added; C, verapamil; D, adrenergic inhibitors; and E, rinse. Concentrations added same as in 2-L. 4-L; A, 100μl added (50mg/ml solution); A, second addition of A; TTX added, same concentration as 1, 2, and 3. R=right atrium. L=left atrium.

vironmental control of *G. toxicus* and hence possible control of the fish poisoning problem associated with ciguatoxin and other polyethers: for example, understanding the growth stimulant, displacement of algae favourable to *G. toxicus* growth with algae inhibitory to *G. toxicus*, and control of the growth factors coming from the inlet tunnel into the harbor. In Waianae Boat Harbor, this means 1, diverting the tunnel outside the harbor to prevent rainfall bringing soil extract; 2, determination of the life cycle of *G. toxicus*; 3, inhibiting by appropriate compounds

the weak link of the *G. toxicus* life cycle and; 4, assessing the toxic fish level and variability in toxicity. But thus far, the data reveal no biological cyclic effect. This probably results in the continuous presence of *G. toxicus* throughout the year. The food chain concept has been verified in this study: *G. toxicus* growth near *Bryopsis* is eaten or taken in by mullet, kole, manini, etc., then travels up to the carnivores such as ulua and kahala and finally to man. In the case of WBH, the major ciguatera causative fish was mullet. A few mullet revealed *G. toxicus* in the gut smear

Table 5.Results of Crude Fish Extracts in the Guinea Pig Atrium and Mouse Toxicity Assays

Date	Fish	S-EIA pre-extract results	Guinea Pig Assay**					Mouse Toxicity
			Atria	Inotropic	TTX	Verap	Adren	
4/92	Mullet flesh	B & P	L	+	Sl	Sl	-	5
6/92	Mullet flesh	ND	R	+	+	+	-	5
6/92	Mullet gut	B&P	R	Sl	Vsl	Vsl	-	5
4/92	Mullet flesh	Neg	L	-	-	-	-	0
6/92	Mullet flesh	Neg	R	-	+	-	ND	5
6/92	Mullet gut	Neg	R	+	+	Sl	-	5
3/92	Mullet flesh	Pooled all fish	R	+	+	+	-	5
5/92	Mullet flesh	Pooled all fish	L	+	Sl	-	-	
3/92	Mullet gut	Pooled	L	+	+		ND	5
4/92	Mullet gut	Pooled	L	+	-	Sl	-	5
5/92	Mullet gut	Pooled	R	+	+		-	5
4/92	Hawn kole flesh	B	L	Vsl	-	Vsl	-	5
4/92	Hawn kole gut	B	R	Sl	-	Sl	-	5
5/92	Hawn kole flesh	Pooled	R	+	-	Sl	-	5
5/92	Hawn kole gut	Pooled	L	Sl	-	Sl	-	
4/92	Manini flesh	B&P	R	+	-		-	0
5/92	Manini flesh	B&P	L	+	Sl	Sl	-	5
6/92	Manini flesh	B&P	L	Sl	Vsl	Vsl	-	4
6/92	Manini gut	B&P	L	Vsl	Vsl		-	1
4/92	Manini flesh	Neg	L	-	-		-	2
5/92	Manini flesh	Neg	L	+	Sl	Sl	-	5
6/92	Manini flesh	Neg	L	+	Sl	Sl	-	5
6/92	Manini gut	Neg	L	+	Sl		-	5
	Manini gut	Pooled					-	
4/92	Manini gut	Pooled	L	+	Sl		-	5
5/92	Manini gut	Pooled	R	-	-		-	5
5/92	Papio flesh	Pooled	R	-	-		-	2
5/92	Papio gut	Pooled	L	Vsl	-		-	5

*S-EIA Results: B, borderline; P, positives; ND, no data; Neg, negative; Pooled, pooled extracts of the same species (negative, borderline and positive S-EIA results)
**Atria: L, left; R, right; TTX, tetrodotoxin; Verap, verapamil, Adren, adrenergic; Sl, slight; Vsl, very slight; +, positive; -, negative (no response)

analysis. It is also suggested that until the *G. toxicus* is diminished, herbivores and carnivores in WBH should not be consumed. The *only* safe fish have been the akule and halalu and they are being caught and eaten by recreational fishermen without ciguatera outbreaks. These findings are essentially similar to those reported for Puako, Hawaii (Hokama et al.,1993), except for differences in the presences of algae species associated with *G. toxicus*. Since the major outbreak in 1991, no other reports of toxicity due to WBH fish have been observed to date. This is to be expected, since the species of potentially toxic fish in WBH have been continuously monitored and reported

to the public by the Department of Health. These species include the mullet and all herbivores and carnivores within WBH. These reports of warning also include the presences of *G. toxicus* among *Bryopsis*.

The guinea pig atrial analysis revealed at least 2 toxins in the fish obtained from WBH. These included a ciguatoxin-like (inotropic response inhibited by TTX) and a maitotoxin-like (inotropic response inhibited by verapamil). The *G. toxicus* extract showed that it contained mostly ciguatoxin-like toxin. In addition, a new sodium channel inhibitory toxin(s) has been noted and is being studied.

ACKNOWLEDGEMENTS

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